Desiccation Tolerance and Cryopreservation of In Vitro Grown Blueberry and Cranberry Shoot Tips

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Abstract

In vitro grown shoot tips of two cranberry (*Vaccinium macrocarpon*) cultivars, Wilcox (PI 614079) and Franklin (PI 554998) and three blueberry (*Vaccinium corymbosum*) cultivars, Berkeley (PI 554883), O'Neal (PI 554944) and Brigitta Blue (PI 618166) from the tissue culture collections of the USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis, were tested for recovery from desiccation and following cryopreservation using three protocols. Cold acclimated, encapsulated and sucrose-pretreated blueberry cultivars were tolerant of desiccation under laminar flow of up to 7h while cranberry cultivars were very sensitive to drying by 3h. Cryopreservation screening followed 2 weeks of alternating-temperature cold acclimation. The three blueberry cultivars cryopreserved with the encapsulation-dehydration technique (ED) produced 83% to 92% regrowth. PVS2 vitrification (VIT) (33% to 87%) and controlled rate cooling (CC) (50% to 67%) were also successful for some blueberries. The cranberry cultivars had poor (5% to 37%) recovery with all three techniques and will require further study to improve recovery after desiccation and cryopreservation.

INTRODUCTION

The genus *Vaccinium* L. is diverse and includes a broad range of small fruit crops (blueberry, lingonberry, cranberry) that are high in antioxidant compounds (Zheng and Wang, 2003). The United States Department of Agriculture, Agricultural Research Service (USDA-ARS), National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, preserves about 200 genotypes of cranberries and more than 1500 accessions of blueberries, from about 80 taxa including subspecies and cultivars that originate from 34 countries (Boches et al., 2006)

Cryopreservation, the storage of living materials in liquid nitrogen, is now widely available as a solution for the long-term storage of vegetatively propagated plants (Engelmann, 2004; Reed et al., 2005). Specific cryopreservation techniques are available for a wide range of crop species (Razdan and Cocking, 1997; Reed, 2008), but there is very little information available for *V. corymbosum* L. (blueberry) and none for *V. macrocarpon* Aiton (cranberry). Earlier studies in our laboratory determined freezing rate and cold acclimation requirements for controlled rate cooling of *V. corymbosum, V. uliginosum* L. and *V. ovatum* Pursh. (Reed, 1989). Cold acclimation for 5 weeks increased *V. corymbosum* shoot tip recovery to 58% compared to 26% for the control. Recovery of *V. corymbosum* was significantly better with a 0.1°C/min cooling rate after 0 to 7 weeks cold acclimation than with more rapid cooling. The other species, however, were less successful. The only other report of *Vaccinium* cryopreservation is with cell cultures of *Vaccinium pahalae* Skottsberg that were successfully cryopreserved with the encapsulation-dehydration technique (Shibli et al., 1999).

Desiccation/dehydration tolerance is a key factor for success in most cryopreservation protocols. Cold acclimation can increase cold and desiccation tolerance.

Non-acclimated *Zoysia* Willd. and *Lolium* L. shoot tips encapsulated and air desiccated to 20% moisture content all died; cold acclimated *Lolium* shoot tips could be dehydrated to 20% moisture content without losing viability while *Zoysia* viability declined to 60% at that moisture content with 60% recovery following cryopreservation (Chang et al., 2000). Cold acclimation is also effective for conditioning other temperate plants (Scottez et al., 1992). Desiccation can also be accomplished by osmotic stress. A pretreatment on medium with a high sucrose concentration promotes intracellular water reduction by osmosis and may also contribute to protection of plasma membrane integrity (Plessis et al., 1993).

In many cases scientists begin cryopreservation studies by testing all of the parameters for a cryopreservation protocol. This is a time consuming process that may not result in a useable protocol. Several recent studies show that often existing techniques can be directly applied to additional plant species with few, if any, modifications (Reed et al., 2003; Reed et al., 2006; Uchendu and Reed, 2008). Many new techniques, and improvements of old techniques, were developed since the first study of *Vaccinium* cryopreservation. By initially utilizing well-established protocols, it may be possible to determine a technique that shows promise and then optimize it for the plant of interest. This strategy could greatly reduce the time required to optimize a method for safe storage of important plant materials in liquid nitrogen (Reed, 2001).

The objectives of this study were to evaluate the desiccation tolerance of blueberry and cranberry cultivars and to determine if one of the three most commonly used cryopreservation techniques was suitable for storage of these cultivars.

MATERIALS AND METHODS

Plant Material

Plantlets from three in vitro grown blueberry (*V. corymbosum*) northern highbush cultivars, Berkeley (VAC 849.001, PI 554883), and Brigitta Blue (VAC 1312.001, PI 618166), and a southern highbush blueberry, O'Neal (VAC 312.001, PI 554944) and two cranberry (*V. macrocarpon*) cultivars, Wilcox (VAC 1299.001, PI 614079) and Franklin (VAC 743.001, PI 554998), were multiplied on woody plant medium (WPM) (Lloyd and McCown, 1980), with 2 $mg \cdot L^{-1}$ zeatin, 30 $g \cdot L^{-1}$ sucrose, 3 $g \cdot L^{-1}$ agar (Difco, Detroit, MI), 1.25 $g \cdot L^{-1}$ Gelrite (Phyto Technology Lab., Shawnee Mission, KS) at pH 5.2. Standard growth room conditions were 25 °C with a 16 h light / 8 h dark photoperiod (40 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Reed and Abdelnour-Esquivel, 1991).

Cold Acclimation (CA)

Micropropagated plantlets of all cultivars were incubated under 22°C with 8h low light (10 $\mu E \cdot m^{-2}$)/-1 °C 16 h dark CA conditions for two weeks (Reed, 1990). Shoot tips (0.8-1 mm) with attached leaf primordia were excised from cold-acclimated plantlets and further exposed to CA conditions for 48h on pretreatment medium with 5% dimethyl sulfoxide (DMSO) and 0.8% agar before cryopreservation.

Desiccation Procedure

The ability of shoot tips of blueberry and cranberry cultivars to retain viability under water stress conditions was tested. Shoot tips were excised from 2-week cold acclimated, in vitro grown plantlets and encapsulated in alginate beads. The samples were precultured in 0.75 M sucrose for 19h. Samples were further exposed to laminar air flow (0.6 $m \cdot sec^{-1}$ air flow at ~22°C and 35±2% relative humidity) and allowed to air-dry from 3 to 7h. Five beads of each cultivar were plated as controls before air-drying the samples. Beads were rehydrated in liquid medium for 10 min after each selected drying period and transferred to culture medium (WPM) in standard growth room conditions. Regrowth assessment was done after 6 weeks of culture.

Fresh Weight (Fw)/Dry Weight (Dw)

Four samples of 20 dried empty beads were used to determine the percentage moisture content of beads after dehydration. This was calculated as (Fresh Wt - Dry Wt)/Fresh Wt x 100. Fresh weight is the weight of beads after pretreatments and before dehydration under the flow hood. Dry weight is the oven dried weight of beads (102°C for 24h).

Cryopreservation Techniques

Shoot tips were plunged into liquid nitrogen (-196°C) for cryopreservation following encapsulation dehydration (ED), PVS2-vitrification (VIT) and controlled rate cooling (CC). Sample vials were rapidly rewarmed in 45°C water for 1 min and in 25°C water for an additional minute before the samples were rinsed and plated on recovery medium.

Encapsulation-Dehydration Procedure (ED)

The protocol described for pear shoot tips was used (Dereuddre et al., 1990). Shoot tips were dissected from plantlets, temporarily cultured on an agar plate and immediately encapsulated in beads of 3% (w/v) low viscosity alginic acid (Sigma chemical Co, USA) in liquid MS medium (Murashige and Skoog, 1962) without calcium at pH 5.7. The beads with shoot tips were polymerized in MS medium with 100 mM $CaCl_2$ and 0.4 M sucrose for 20 min. Encapsulated shoot tips were preconditioned in liquid MS with 0.75 M sucrose on a rotary shaker (50 rpm) for 18h. Beads were blotted dry on sterile filter paper, transferred to an empty sterile glass Petri dish and dehydrated for 6h (~22% bead moisture content) in a laminar-flow hood. Twenty dehydrated beads were placed in 1.2 ml cryovials (10 beads per cryovial) and plunged into liquid nitrogen (LN). The alginate beads were rehydrated in liquid MS for 10 min after rewarming as described above and before being transferred onto regrowth medium (WPM) in 24-cell tissue culture plates (Costar, Cambridge, Mass.). The controls were prepared by rehydrating and planting 5 beads for each cultivar after 18h of preconditioning in liquid MS with 0.75 M sucrose and after 6h of dehydration under the laminar flow hood.

Vitrification Procedure (VIT)

The PVS2 vitrification procedure (Sakai et al., 1990) was followed. Shoot tips excised from cold acclimated plantlets were pretreated for 48h on MS agar plates containing 5% (v/v) dimethyl sulfoxide [DMSO (Sigma-Aldrich Co., St Louis, MO)] with 3.5 g agar and 1.75 g/L Gelrite under CA conditions. Shoot tips were transferred into 1.2 ml cryovials and treated with 1 ml loading solution (LS) (2 M glycerol in 0.4 M sucrose solution (v/v), pH 5.8) for 20 min at 25°C. LS was removed and 1 ml PVS2 cryoprotectant solution [30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS with 0.4 M sucrose (v/v)] was added to cryovials for 20 min at 25°C, then vials were plunged into LN. The shoot tips of each cultivar were rinsed in 1.2 M sucrose liquid MS and planted on recovery medium as controls after pretreatment with PVS2. Rewarming was done as described above. The PVS2 solution was diluted with liquid MS medium containing 1.2 M sucrose and shoot tips were drained on filter paper and planted on the recovery medium.

Controlled Rate Cooling (CC)

Controlled rate cooling was done following the protocol of Reed (1990). Shoot tips were excised and placed on MS agar plates containing 5% (v/v) dimethyl sulfoxide [DMSO (Sigma-Aldrich Co., St Louis, MO)] with 1.75 $g \cdot L^{-1}$ Gelrite and 0.8% agar for 48h. The shoot tips were transferred to 1.2 ml plastic cryovials containing 2 drops of liquid MS medium on ice. Two drops of the PGD cryoprotectant [polyethylene glycol (10% w/v PGD (MW 8000), glucose, and DMSO in liquid MS medium] (Finkle and Ulrich, 1979) was added to the cryovials every 2 min for 6 min then 4 drops every 2 min over a 30 min period. Cryovials were equilibrated for 30 min on ice. Excess

cryoprotectant was decanted to 1 ml before loading samples into the programmable freezer (0.1/min to -9°C for exotherm; 0.1/min to -40°C). The unfrozen controls were left on ice until the exotherm, after which the PGD was removed, samples were rinsed in liquid MS and the shoot tips were planted on recovery medium. The frozen samples in cryovials were plunged in LN at the end of the programmed run. Samples were thawed as above, rinsed in liquid MS medium and planted on recovery medium.

Statistics

Assessments of the recovery of shoot tips were made 6 weeks after rewarming. Shoot survival, proliferation, leaf expansion and greening were considered in order to determine successful recovery from cryopreservation. Each experiment was replicated three times. Each experiment included 20 cryopreserved shoots (n = 60 shoots for each treatment) and 5 unfrozen, but cryoprotected shoot tips per treatment (n = 15 shoots for each treatment). The data is presented in graphs as percentages. ANOVA and Duncan's Multiple Range Test ($P \leq 0.05$) using SAS version 9.1 was applied to data analysis (SAS, 2003).

RESULTS AND DISCUSSION

Effect of In Vitro Desiccation

There were significant differences between the encapsulated blueberry and the cranberry shoot tips in response to desiccation under laminar flow ($P < 0.0001$). All the blueberry cultivars recovered at a high rate and significantly better than the cranberries. Blueberry cultivars retained >80% viability with up to 7h desiccation (Fig. 1A). The regrowth of cranberry cultivars declined sharply with 3 or more hours of desiccation, indicating that they are highly desiccation sensitive (Fig. 1B). Water content of the beads declined to 20% from the initial 90% after 7h dehydration (Fig. 1).

Effect of Cryopreservation Protocols on the Recovery of Blueberry Cultivars

The blueberry cultivars tested had significant differences in recovery with each protocol, but in most cases produced moderate to high regrowth (Fig. 2). The ANOVA showed an interaction of genotype by technique ($P < 0.0001$). For 'Brigitta Blue' all three protocols produced good regrowth but ED and VIT were significantly better than CC. For 'Berkeley' ED was significantly better than VIT and CC but VIT and CC were not significantly different. 'O'Neal' regrowth was also best with ED, lower with CC and lowest with VIT.

The only other study of *Vaccinium* shoot tip cryopreservation studied CC. In that study, 5 weeks cold acclimation prior to controlled rate cooling at 0.1°C/min produced some regrowth for the three species tested. One *V. corymbosum* accession had 58% recovery, but only 10 to 40% recovery was reported for shoot tips of *V. ovatum*, *V. uliginosum* and another *V. corymbosum* cultivar in that study (Reed, 1989). Our current CC results with *V. corymbosum* cultivars CA for 2 weeks were in the same range (51-67%) as *V. corymbosum* in the earlier study.

The northern and southern highbush blueberries all responded well (>80% regrowth) to cryopreservation with the ED technique that relies on osmotic and air drying to condition the shoot tips (Fig. 2). The northern highbush blueberries are naturally adapted to areas with cold winters while southern highbush blueberries are adapted to hot summers (Trehane, 2004). The natural adaptation of these plants to desiccating conditions allows us to manipulate them in culture and take advantage of their natural desiccation tolerance. Additional preculture steps before cryopreservation using VIT or CC would likely improve blueberry regrowth. The high recovery of encapsulated blueberry shoot tips following desiccation in laminar flow is attributable to the combination of cold acclimation and desiccation in 0.75 M sucrose applied in this study (Fig. 1A). This CA-sucrose desiccation combination is also the prelude to ED cryopreservation that resulted in very good regrowth (Fig. 2). The VIT and CC procedures for 'Berkeley' and 'O'Neal'

might show improved results with increased CA or a sucrose pretreatment step prior to cryopreservation. Cryobiologists often apply cold acclimation as a pretreatment strategy to improve tolerance to desiccation and increase survival following cryopreservation (Chang and Reed, 2000; Dereuddre et al., 1990; Reed, 1990). Cold acclimation is known to enhance accumulation of polyamines and special proteins including dehydrins, and to up-regulate the expression of low temperature genes in *Vaccinium* (Naik et al., 2007). Cold acclimation may also promote stability of membrane structure through the action of dissolved solutes that may form hydrogen bonds with hydrophilic molecules present in the cells (Taylor, 1987).

Effect of Cryopreservation Protocols on the Recovery of Cranberry Cultivars

Both cranberry cultivars had significantly less regrowth than the blueberry cultivars ($P < 0.0001$). Recovery ranged from 8% to 37% following the three cryopreservation techniques (Fig. 3). 'Franklin' had the best recovery (37%) with the VIT procedure, but recovery from CC and ED was significantly lower. 'Wilcox' regrowth with ED and VIT was not significantly different. VIT and CC were not significantly different and all recovery of Wilcox was < 20%. Neither cranberry cultivar reached the 40% recovery recommended for germplasm storage (Reed, 2001; Reed et al., 2005).

The key role of desiccation tolerance in cryopreservation is clearly illustrated by the differential recovery of these two *Vaccinium* species to desiccation and cryopreservation. Blueberry cultivars were tolerant to desiccation even at 20% moisture content whereas the cranberry cultivars were not (Fig. 1). Consequently, blueberry cultivars had good regrowth with all three cryopreservation techniques (Fig. 2) while cranberry cultivars performed poorly (Fig. 3). This result demonstrates that a reduction in freezable intracellular water is one of the key requirements for the cryopreservation of *Vaccinium* species and cultivars. This parallels the results obtained with many other plants including *Cichorium intybus* L. where reducing residual water to about 20% significantly improved recovery following cryopreservation (Vandenbussche et al., 1993). Paul et al. (2000) found that 6h dehydration to a 21% water content resulted in 83.7% recovery of ED cryopreserved shoot tips of apple.

Cranberries are a wetland-adapted crop species and can thrive even in flooded soils (Eck, 1990). This natural wet adaptation indicates that the plants may require high water content for growth and would require a slower dehydration procedure, perhaps in a stepwise manner, in order for them to tolerate desiccation and cryopreservation. To improve cranberry desiccation tolerance we propose a preculture in sucrose or abscisic acid (ABA). ABA is known to play a significant role in plant water balance and in the adaptation of plants to environmental stresses (Bravo et al., 1998). Preculture in sucrose and ABA medium enhanced desiccation and freezing tolerance in *Gentiana scabra* Bunge var. *buergeri* Maxim. axillary buds (Suzuki et al., 2006). In addition, pretreatment with increasing concentrations of sucrose prior to PVS2 exposure could be explored as a dehydration technique for cranberries (Sakai et al., 1990).

CONCLUSIONS

This study provides additional information on the cryopreservation of *Vaccinium* spp. We determined that in vitro grown blueberry cultivars responded well to the stresses imposed by desiccation and produced high recovery when cryopreserved; whereas the cranberry cultivars were desiccation sensitive and will require additional manipulation to reduce cellular water content. Blueberry cultivars that were cold acclimated for 2 weeks could be cryopreserved by any of the three techniques, but ED was excellent for all three cultivars tested. Cranberry cultivar recovery was very low following cryopreservation by each of the three techniques, and the protocols will require modification before cranberries can be stored for long-term preservation.

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Figures

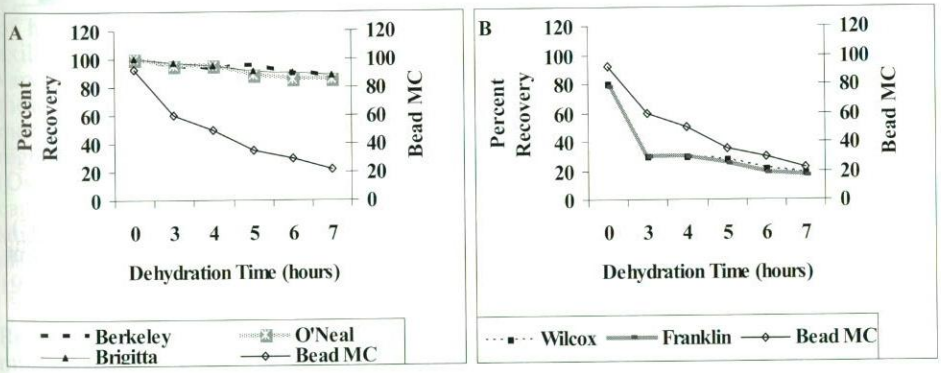

Fig. 1. Percent recovery and moisture content (MC) of blueberry (A) and cranberry cultivars (B), 6 weeks after encapsulation in alginate beads, 18h in 0.75 M sucrose and 0 to 7h desiccation under laminar flow without exposure to liquid nitrogen. Beads were rehydrated and plated on recovery medium after each time period.

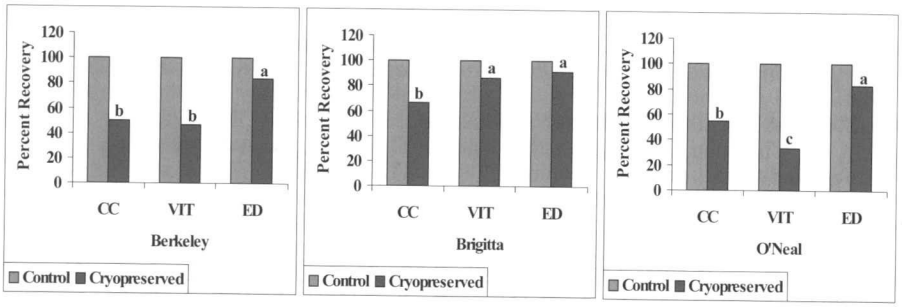

Fig. 2. Percentage recovery of blueberry cultivars following controlled rate cooling (CC), vitrification (VIT) and encapsulation dehydration (ED). Means with different letters are significantly different at $p \leq 0.05$

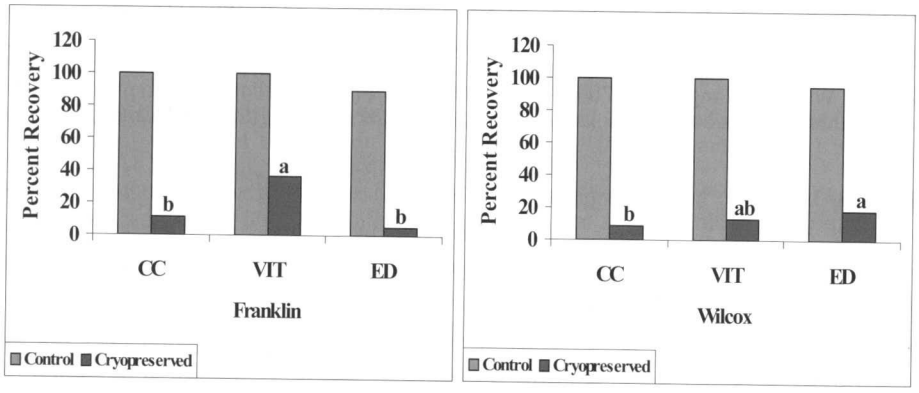

Fig. 3. Percentage recovery of cranberry cultivars following controlled rate cooling (CC), vitrification (VIT) and encapsulation dehydration (ED). Means with different letters are significantly different at $p \leq 0.05$.